

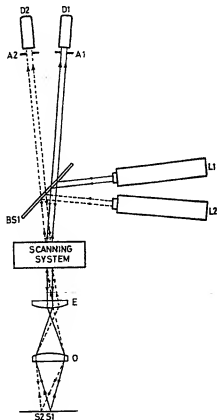


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>G02B 21/00, G01N 21/64</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/01966</b> <b>(43) International Publication Date:</b> 6 February 1992 (06.02.92)
<b>(21) International Application Number:</b> PCT/GB91/01176 <b>(22) International Filing Date:</b> 16 July 1991 (16.07.91) <b>(30) Priority data:</b> 9015793.4 18 July 1990 (18.07.90) GB <b>(71) Applicant (for all designated States except US):</b> MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> AMOS, William, Bradshaw [GB/GB]; 54 Cavendish Avenue, Cambridge CB1 4UT (GB). <b>(74) Agents:</b> NASH, Keith, Wilfrid et al.; Keith W Nash & Co, Pearl Assurance House, 90-92 Regent Street, Cambridge CB2 1DP (GB).	<b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. <b>Published</b> <i>With international search report.</i>	

**(54) Title:** CONFOCAL SCANNING OPTICAL MICROSCOPE**(57) Abstract**

A confocal scanning optical microscope in which a specimen under test is simultaneously scanned with two distinct spots or slits of illumination (S1, S2), and two output beams emitted from the specimen due to reflection or fluorescence are descanned and passed to separate stationary confocal apertures (A1, A2) and detectors (D1, D2).



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU <sup>+</sup>	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

<sup>+</sup> It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

- 1 -

Title    Confocal Scanning Optical MicroscopeField of the invention

This invention relates to an optical confocal scanning microscope.

Background to the invention

U.K. Patent Publication No. 2 184 321A discloses a confocal scanning optical microscope which is especially for the study of fluorescent or reflecting specimens. The instrument depends upon the focussing of light upon a single spot scanned over the specimen, which illuminated spot, after de-scanning, is imaged on a confocal aperture in front of a detector.

In the case where an image is to be formed of fluorescence from a specimen, the wavelength of the light directed on to the specimen is selected in such a way as to excite fluorescence. The emitted light is separated from the exciting light by a suitable beam splitter and is passed through wavelength-selective filters in such a way that the detector responds only to the light emitted by fluorescence. Instruments based on this design are commercially available. They contain a provision for subdividing the emitted light into beams of different wavelength ranges by a suitable beam splitter and filters. After this division, two dyes can be employed which emit different colours of fluorescence which can be distinguished at two detectors. Alternatively, a

- 2 -

reflectance image can be obtained at the same time as a fluorescence image by the use of suitable beam-splitters, in accordance with accepted optical practice.

The prior art instruments work satisfactorily but all confocal scanning microscopes which rely on the use of a single scanning spot suffer from the defect that all the spectral selectivity of the system lies in the separation of the emitted or reflected beam into fractions of different wavelength. If there is considerable overlap between the fluorescent emission spectra of two dyes, they cannot be distinguished. For example, Bacallao et al comment in the Handbook of Confocal Microscopy, Plenum Press, 1990, that the commonly-used dyes fluorescein and rhodamine cannot be separated effectively in a system of this type. In order to achieve acceptable separation, it is necessary to vary the wavelength of excitation. This can be done by changing from one type of laser light to another, of spectrally different properties. First an image is obtained by operating the system with one type of excitation, and then a second image is obtained with a different type of exciting beam. This operation is slow and cumbersome.

Awamura, Ode and Yonezawa have described a microscope in which red, green and blue laser beams are scanned independently over the specimen, and the reflected beams are separated by dichroic filters and each executes a scanning motion over one of three separate linear CCD detector arrays. The description was published in the Proceedings of SPIE, The International Society for Optical Engineering (1987) Volume 765 pp 53-60. In principle, the system of Awamura et al might be used as a fluorescence microscope. It would then allow more than

- 3 -

one type of dye to be excited in rapid succession during each line scan. However, in the case of two dyes with identical emission spectra, or a single ratiometric dye where the emission spectrum was to be monitored in a single waveband, the system of Awamura et al offers no advantage over that of White (U.K. Patent Application No. 2 184 321A), since neither system is capable of separating the two emission signals.

#### The invention

According to the present invention, there is provided a confocal scanning optical microscope comprising:

- an optical scanning system;
- means for simultaneously generating two or more input beams of optionally different spectral composition and of differing orientations such that after passage through the scanning system a specimen under test is scanned with two or more distinct and separate elemental areas of illumination; and
- two or more detectors respectively for receiving two or more output beams after de-scanning by the scanning means, each detector receiving an output beam substantially restricted to output light derived from one of the illuminated elemental areas.

The invention allows for two or more microscope channels having different excitation wavebands but identical emission wavebands, in order to make possible excitation ratio image measurements according to accepted practice.

- 4 -

The invention also allows for two or more microscope channels having identical excitation wavebands but different emission wavebands, in order to make possible emission ratio image measurements according to accepted practice.

The present invention is thus applicable to many kinds of scanning optical microscope. It provides a means by which two or more spectrally distinct exciting spots or bars can be scanned together over the specimen during each sweep of the scanning system. The emission from each spot is passed individually and separately to a stationary confocal aperture leading to a detector, there being at least one aperture and detector for each spot.

The emitted beam from each spot, due to specimen fluorescence or reflection, may be filtered spectrally or subdivided between detectors in accordance with established practice, or may be passed unselectively to the detector. It is thus possible to obtain, within a single scanning cycle, two or more complete images, each of which may differ in both excitation and emission waveband from the other images.

The invention may be considered as "a multiplexed optical system" because it involves two or more sets of independent but near-parallel beam paths passing through the same scanning system and objective lens, the optical paths being multiplexed in the literal sense of being folded together.

#### Description of embodiment

Further features and advantages of the invention will be

- 5 -

apparent from the following description of embodiments, making reference to the accompanying drawings, in which:-

Figure 1 is a schematic diagram of a confocal scanning microscope incorporating the multiplexed optical system of the present invention;

Figure 2 is a schematic diagram showing an alternative and preferred optical arrangement for the upper part of Figure 1; and

Figure 3 is a schematic diagram showing an optical means by which several beams of different spectral properties may be obtained from a single (e.g. a multiline) laser, for use in the present invention.

Referring to the drawings, the present invention provides an optical assembly which allows a number of independent optical channels to be used simultaneously for excitation in a laser confocal scanning microscope with an extended emission beam path, but is not restricted in application to this kind of microscope. The invention can be applied to confocal microscopes in which a bar or slit of light is scanned over the specimen as well as to those in which a single spot is scanned.

In Figure 1, to simplify the diagram, only two independent light paths are shown, but there is no restriction on number in practice.

Light from two lasers, L1 and L2, with different spectral qualities, is directed on to a beam splitter BS1. The

- 6 -

two beams are at a slight angle to each other, which angle is exaggerated in the diagram for the sake of clarity. The two beams are reflected into a scanning system as shown, which produces an angular scan of both beams simultaneously. The angular separation of the beams is maintained throughout the scan, and results, after passage through suitable microscope optics, typically an eyepiece E and an objective O, in the formation of two distinct moving spots of light S1 and S2 on the specimen.

Light is emitted from the specimen at S1 because of reflection or fluorescence and a portion of this emitted light passes back through the optical system, is descanned, i.e. reconverted into a stationary beam by the scanning system, passes through the beam splitter BS1 and falls on a confocal aperture A1 leading to a detector D1. Light from S2 passes through the optical system along a similar but distinct path and falls upon detector D2. The preferred angular separation is the smallest possible consistent with a satisfactory separation of the optical channels. To allow image registration, the small difference in time between the scanning of a given point in the specimen by the spots corresponding to S1 and S2 may be compensated by suitable conventional electronic means, for example by image processing software. It is not essential to the functioning of the system that the two or more spots should lie upon the same scan line.

In the preferred embodiment of Figure 2, the scanning device and microscope are not shown in the figure, but should be taken to be the same as in Figure 1. Beams from lasers L1 and L2 again pass at a small angle on to the beam splitter BS1. The returning beams, after passing through beam splitter BS1, pass to a second beam



- 7 -

splitter BS2, which has dichromatic properties, so that most of the light in one of the beams, B1, passes through to confocal aperture A1 and thus to detector D1, while the other beam B2 is preferentially reflected to A2 and D2. This modification is preferred as it allows the use of the second beam splitter BS2 to achieve a selection of emission wavelengths, and also may be implemented by only slight modification of existing instruments. The separation of the emitted beams by wavelength may be improved by the addition of wavelength-selective filters F1 and F2.

The aiming of the emission beams, each on to the appropriate aperture A1 or A2, may conveniently be achieved by the use of mirrors (not shown) interposed between BS2 and the detectors D1 or D2.

Additional mirrors and dichromatic reflectors may provide convenient means of achieving an appropriate angle between the input beams L1 and L2. For example, Figure 3 illustrates one of many possible means by which light from a single multiline laser L may be separated into beams of different spectral composition and angle.

In this case, a parallel-sided block B of glass or other transparent material is used to produce a small lateral separation of the beams according to wavelength. The angle between the beams is then adjusted by passing them through a prism P, where they undergo different angular deviations because of the dispersing power of the prism. By appropriate orientation of the prism, parallel beams, each corresponding to a single wavelength, are generated, which converge towards the beam splitter BS1. The angle of convergence is determined by the angle of the prism and

- 8 -

its refractive index and dispersive power. In the diagram, the solid line S indicates a beam at a shorter wavelength, which is more strongly refracted than the beam, shown by the dashed lines D, corresponding to light of a longer wavelength.

Various modifications of the above-described and illustrated arrangements are possible within the scope of the invention hereinbefore defined.

Claims

1. A confocal scanning optical microscope comprising:
  - an optical scanning system;
  - means for simultaneously generating two or more input beams of optionally different spectral composition and of differing orientations such that after passage through the scanning system a specimen under test is scanned with two or more distinct and separate elemental areas of illumination; and
  - two or more detectors respectively for receiving two or more output beams after de-scanning by the scanning means, each detector receiving an output beam substantially restricted to output light derived from one of the illuminated elemental areas.
2. A microscope according to claim 1, in which the two or more beams define two or more microscope channels having different excitation wavebands but identical emission wavebands.
3. A microscope according to claim 1, in which the two or more beams define two or more microscope channels having identical excitation wavebands but different emission wavebands.
4. A microscope according to claim 1, in which the two or more beams define two or more microscope channels having different excitation wavebands and different emission

- 10 -

wavebands.

5. A microscope according to any of claims 1 to 4 in which the emission from each elemental area is passed individually and separately to a stationary confocal aperture leading to a detector, there being at least one aperture and detector for each area.

6. A microscope according to any of claims 1 to 5, in which the elemental areas are in the form of spots or bars.

7. A microscope according to claim 6, being a slit-scanning confocal microscope in which the bars are generated by images of slits.

8. A microscope according to any of claims 1 to 7, in which the input beams are set at a small angle to one another.

9. A microscope according to any of claims 1 to 8, in which the input beams are obtained by dividing the light from a multiline laser.

10. A microscope according to any of claims 1 to 9, in which a beam splitter provides for separation of output beams.

11. A microscope according to any of claims 1 to 9, in which separation of the output beams by wavelength is enhanced by one or more wavelength-selective filters.

12. A microscope according to any of claims 1 to 11, in which the distinct input beams are obtained by passing a

- 11 -

multiple wavelength beam through a prism.

13. A microscope according to any of claims 1 to 12, in which difference in path length times relating to the different scanned elemental areas is compensated for electronically.

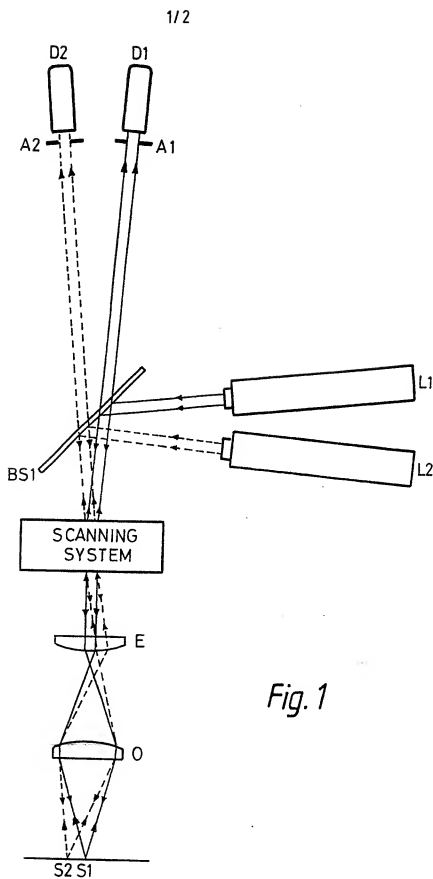
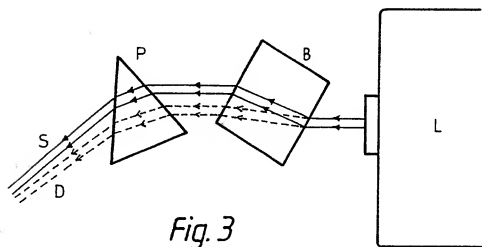
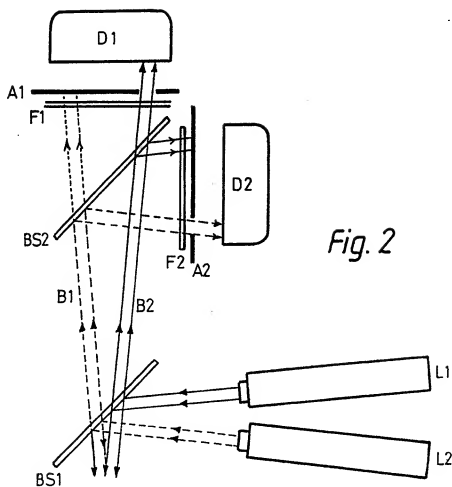


Fig. 1

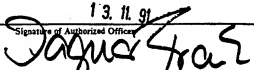
2/2



## INTERNATIONAL SEARCH REPORT

International Applic. No

PCT/GB 91/01176

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl.5                      G 02 B 21/00                      G 01 N 21/64		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl.5	G 02 B                      G 01 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	US,A,3918812 (D.M. HOLM) 11 November 1975, see abstract; figures 2,3; column 4, line 52 - column 6, line 40 ---	1,9,12
A	GB,A,2184321 (MEDICAL RESEARCH COUNCIL) 17 June 1987, see the whole document (cited in the application) ---	1
A	DE,A,3831880 (JENAOPTIK JENA) 3 May 1989, see the whole document ---	1
A	US,A,4893008 (Y. HORIKAWA) 9 January 1990, see column 1, lines 5-9; column 2, lines 29-65; column 3, line 67 - column 7, line 44; figures 3-5 ---                                      -/-	1,5,6,7 ,10,11
<p><sup>10</sup> Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
10-10-1991	13. 11. 91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE		

Mme Dagmar FRANK



III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
A	Cytometry, volume 2, no. 4, 1982, (US) J.A. Steinkamp et al.: "Three-color fluorescence measurements on single cells excited at three laser wavelengths", pages 226-231, see figures 1-3; page 227 - page 228, column 2, line 16 -----	1,2,4-7 ,10,11
A	US,A,4284897 (I. SAWAMURA) 18 August 1981, see the whole document -----	1,10,11

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9101176

SA 49462

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/11/91.  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3918812	11-11-75	AU-A- 6864274	06-11-75
		BE-A- 814460	02-09-74
		CA-A- 1020234	01-11-77
		DE-A- 2422016	28-11-74
		FR-A, B 2229063	06-12-74
		GB-A- 1448789	08-09-76
		JP-A- 50016597	21-02-75
GB-A- 2184321	17-06-87	None	
DE-A- 3831880	03-05-89	None	
US-A- 4893008	09-01-90	JP-A- 63306413	14-12-88
US-A- 4284897	18-08-81	JP-C- 1154952	15-07-83
		JP-A- 53135660	27-11-78
		JP-B- 57049888	25-10-82
		DE-A, C 2818841	09-11-78